UNIVERZITA KARLOVA V PRAZE

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

Katedra biochemických věd

HODNOCENÍ ANTIAROMATÁZOVÉ AKTIVITY ISOFLAVONOIDŮ OBSAŽENÝCH V BIOTRANSFORMOVANÉ SOJI

ve spolupráci s

UNIVERSIDADE DO PORTO

FACULDADE DE FARMÁCIA

Laboratório de Bioquímica

Vedoucí diplomové práce: Prof[a]. Dr[a]. Natércia Teixeira

Prof[a]. Dr[a]. Georgina Correia da Silva

Doc. Pharm.Dr. Tomáš Šimůnek, Ph.D.

Porto 2010

Lucie Koubková

CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRALOVE

Department of Biochemistry

THE EVALUATION OF ANTIAROMATASE ACTIVITY OF ISOFLAVONES FROM BIOTRANSFORMED SOYA

in cooperation with

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„Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem čerpala při zpracování, jsou uvedeny v seznamu použité lieratury a v práci řádně citovány.“

Porto, 2010 Lucie Koubková

ABSTRAKT

Univerzita Karlova v Praze
Farmaceutická fakulta v Hradci Hrálové
Katedra biochemických věd

Kandidát: Lucie Koubková
Školitel: Prof[a]. Dr[a]. Natércia Teixeira
Prof[a]. Dr[a]. Georgina Correia da Silva
Doc. Pharm.Dr. Tomáš Šimůnek, Ph.D.

Název diplomové práce: Hodnocení antiaromatázové aktivity isoflavonoidů obsažených v biotransformované soji

Rakovina prsu patří u žen mezi nejčastěji diagnostikované nádory. Přibližně 60% nádorů prsu je tzv. hormon dependentní, mající na svém povrchu estrogenní receptory a vyžadující estrogeny ke svému růstu. Estrogeny jsou schopné podporovat transkripci genů účastnících se buněčné proliferace. Aromatáza je enzym katalyzující poslední krok biosyntézy estrogenů a její inhibice je považována za důležitý cíl v léčbě na estrogenu závislých nádorů prsu. Sója je zdrojem tzv. fytoestrogenů jako jsou genistein a daidzein. O těchto látkách se předpokládá, že by mohly být účinné v prevenci karcinomu prsu, avšak mechanismus jejich působení není plně pochopen. Tato práce je zaměřena na zhodnocení efektu extraktu sóji obsahující genistein a daidzein na aktivitu výše zmíněného enzymu. Výsledky ukázaly, že tento extrakt inhibuje aromatázu na dávce závislým způsobem, jednak v pokusech s placentálními mikrozomy, tak i v případě buněčné linie MCF-7aro. V pokusech s placentálními mikrozomy bylo rovněž prokázáno, že biotransformace extraktu sóji houbou *Aspergillus awamori* neměla žádný vliv na kapacitu indukce inhibice aromatázy. U buněčné linie MCF-7aro byla pozorována redukce proliferace a morfologické změny buněk v závislosti na dávce extraktu po 48-ti hodinové inkubaci. Extrakt sóji se zdá být slibným a účinným prostředkem v inhibici aromatázy. Avšak další studie jsou vyžadovány k detailnějšímu pochopení mechanismu inhibice aromatázy a objasnění, které látky jsou skutečně zodpovědné za inhibiční efekt.

ABSTRACT

Charles University in Prague
Faculty of Pharmacy in Hradec Kralove
Department of Biochemical Sciences

Candidate: Lucie Koubková
Supervisor: Prof[a]. Dr[a]. Natércia Teixeira
Prof[a]. Dr[a]. Georgina Correia da Silva
Doc. Pharm.Dr. Tomáš Šimůnek, Ph.D.

Title of diploma thesis: Evaluation of anti-aromatase activity of isoflavones from biotransformed soya

Breast cancer belongs to the most frequently diagnosed cancers in women. Approximately 60% of breast cancer is hormone-dependent containing estrogen receptors and requiring estrogen for tumour growth. Estrogens are able to promote transcription of genes involved in cell proliferation. Aromatase is responsible for the final step of estrogen biosynthesis and its inhibition has been considered as an important target for the treatment of estrogen-dependent breast cancer. Soya is a source of phytoestrogens, such as genistein and daidzein, which are thought to be effective in breast cancer prevention. However, the mechanisms associated with this effect are not fully understood. In this study we evaluated the effects of a soya extract containing genistein and daidzein on aromatase activity. Our results demonstrated that this extract had the ability to inhibit aromatase in a dose-dependent manner in placental microsomes and also in the MCF-7aro breast cancer cell line. It was also shown that, in the placental microsomes, the biotransformation by fungi *Aspergillus awamori* did not alter the capacity to induce aromatase inhibition. In this cell line it was also observed a dose dependent reduction in cell proliferation and morphological alterations after 48 hours of treatment. This biotransformed extract seems to be quite potent and a promising agent to induce aromatase inhibition, but further studies are required to better understand the mechanism of aromatase inhibition by soya contents and elucidate what kind of compounds are exactly responsible for the inhibitory effect.

CONTENTS

1. INTRODUCTION

1.1 Breast cancer

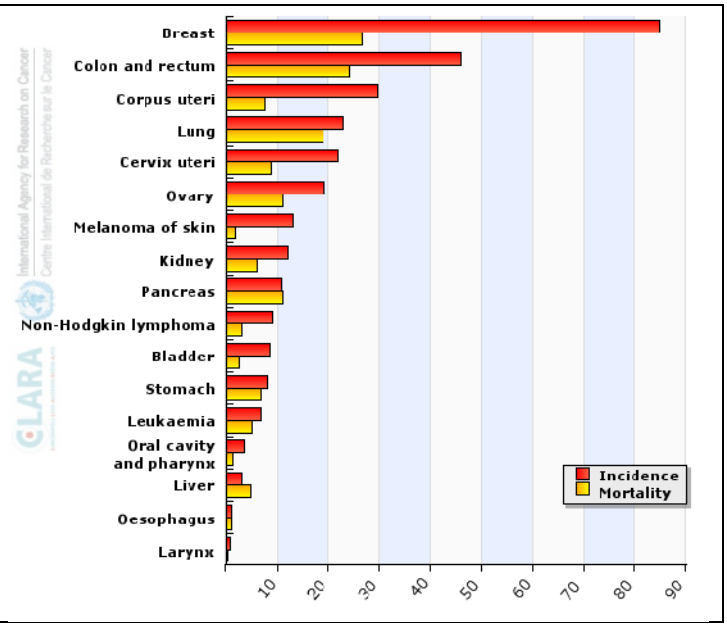
Cancer is the second leading cause of death after the coronary diseases. There are 3.2 million diagnosed cases of cancer every year in Europe (47% occurring in women, 53% in men) and more than 1.7 million deaths from cancer (44% in women, 56% in men) (Ferlay et al., 2007). There is a strong association between cancer risk and age. Due to the aging of the European population these numbers are probably going to increase. It is estimated that by 2015 there will be a 22% increase in the population aged >65 years and a 50% increase in the number of persons aged >80 years old (Boyle and Ferlay, 2005). In 2006 the most common incident cancer was the breast cancer, followed by colorectal and lung cancer.

Breast cancer belongs to the most frequently diagnosed cancers in women. World Health Organization estimates that breast cancer affects one out of every sixteen women in Europe during their lifetime [1]. Breast cancer incidence varied in Europe with lowest rates in Central Europe, Croatia and Slovenia (41 to 64 per 100,000) and highest rates in the Netherlands and Italy (91 per 100,000) (Karim-Kos et al., 2008).

In Czech Republic according to the Institute of Health Information and Statistics there were reported 5884 new cases of breast cancer in 2006, which represents almost 17% of all oncological diseases in women. Although the treatment of breast cancer especially in the very early stages is successful (almost 71% of cases of stage I and II) it remains the most common malignant neoplasm in women leading to death in Czech Republic (figure 1).

Figure 1.

Estimated incidence and mortality in women from Czech Republic (2006); age standardized rate (European) per 100,000 [2]



In the long term the mortality rates has slightly decreased. This reduction is due to the screening programs, which reveal the cancer in the beginning stage, due to better access to health care and also due to new approaches in breast cancer treatment. In addition, the breast cancer incidence has been attributed to the lower use of hormone replacement therapy (Karim-Kos et al., 2008). Despite of the advances in research and treatment, breast cancer remains one of the most serious health problems.

Numerous epidemiological factors are associated to the development of breast cancer. The female sex is considered to be a risk factor. Men can also suffer from this disease, but it represents only 1% of all breast cancers diagnosed. The risk of developing increases with increasing age. The most common occurrence in women is between the 50 and 60 years old being the majority of breast cancer diagnosed after the age of 60 [3]. Lifestyle factors related to energy balance, including dietary behaviour, weight and physical activity, can also contribute to cancer risk. Fuemmeler et al. (2009) carried out a study suggesting that unhealthy behaviours developed early in life and persisted over time may increase the risk of some cancer types, such as premenopausal breast cancer.

The environment is also considered to have an influence on cancer development. Hormonally active agents found in the environment, for example organochlorine pesticides, like dichlorodiphenyltrichloroethane (DDT) and its metabolite dichloro-diphenyldichloroethylene (DDE) are suspected to influence breast cell proliferation by acting as estrogen or by disrupting pathways leading to cell proliferation (Snedeker, 2001). In addition, 5-10% of malignant breast tumours can arise from hereditary predispositions. Mutation in the tumour-suppressor genes BRCA1 or BRCA2 are strong predictors of breast cancer onset. The individuals carrying mutations in one of these genes have a 40-80% chance of developing this disease (Fackenthal and Olopade, 2007). Mutation prevalence varies among different geographic regions and ethnic groups. Other mutations in genes, which encode proteins controlling cell cycle, cell growth and proliferation as well as signalling pathways, contribute to increased risk of cancer development.

It is well established that higher risk of developing cancer is associated with high-dose radiation treatment, increased density of breast tissue or hyperplasia, early menarche (before 12 years) and late menopause (after 50 years), having first child after 30 years, use of oral contraceptives or long-term hormone replacement therapy (more than 5 years) to relieve menopausal problems, excessive drinking of alcohol, obesity and overall unhealthy life style (Russo et al., 2003). There are also some differences between races and ethnicities. White women are more susceptible than African American, but African American women are more likely than white women to be diagnosed with large tumours and distant-stage disease. Other ethnic groups have lower incidence rates (Ghafoor et al., 2003).

1.2 Estrogens

Estrogens are a group of steroid hormones which can easily pass through cell membrane and induce gene expression. They are the primary female sex hormones produced mainly by the adrenal cortex and ovary but also by the corpus luteum and placenta in response to impulses from the hypothalamic-pituitary axis. Some small percentage of estrogen is produced by other tissues such as liver, adrenal gland, mammary gland and adipose tissue.

In fact estrogens include three hormones: estradiol, estriol and estrone. The most potent is estradiol, which also have highest affinity for its receptors and is produced in the large quantities by the ovaries. Estrone is less potent metabolite of estradiol produced from androstenedione in adipose tissue during menopause. Estriol, the third endogenous estrogen originated also from estradiol, is produced by placenta during pregnancy. In non-pregnant women is found in smaller quantities (Chen et al., 2008).

Estrogens influence many physiological processes like regulation of female reproductive organs, proliferation of endometrium and other aspects of the menstrual cycle, promoting formation and maintenance of female secondary sex characteristics. They also play a significant role in the metabolism. By reducing bone resorption estrogens prevent the development of osteoporosis (Hodek et al., 2005). It is well known, that post-menopausal women, whose ovaries ceased estrogen production may start suffering from osteoporosis. In liver cells, estrogens alter the production of proteins that influence cholesterol levels in the blood. There are two types of lipoproteins produced by the liver cells. Low density lipoprotein (LDL) can promote plaques formation on the arteries' wall, whereas high density lipoprotein (HDL) inhibits plaques formation and carries cholesterol back to the liver. Thus estrogen's action on liver cells decreases levels of LDL and increases HDL levels that help to reduce the risk of coronary diseases. They also have an influence on brain functions. For example estrogens have started to be used for treatment of Alzheimer disease in women, as they may affect brain function by avoiding effects of apolipoprotein E and beta-amyloid (Rokyta, 2000).

Although estrogens have many beneficial effects they can also be harmful. By promoting cell proliferation they may contribute to accumulation of spontaneous mutations. Later, especially mutations in tumour suppressor genes or proto-oncogenes can lead to uncontrolled proliferation and the onset of cancer [4].

1.3 Estrogen receptors

Estrogen action on target tissues is mediated through two estrogen receptor subtypes, ER-α and ER-β that belong to the nuclear receptor superfamily of proteins (Ascenzi et al., 2006; Meyer et al., 2009; Vasudevan and Pfaff, 2008; Zilli et al., 2009). ERs have specific expression, but with overlapping distribution in some tissues. ER-α is expressed predominantly in the uterus, pituitary gland, ovaries, liver, kidneys, adrenals, and the mammary glands, while ER-β is expressed more in prostate, bones, ovaries, lungs, and in various parts of the central and peripheral nervous system (Matthews and Gustafsson, 2003; Zilli et al., 2009).

ER-α and ER-β are encoded by distinct genes located on chromosome 6 and 14, respectively. There are several splicing variants of both receptors but their biological function remains unclear. Estrogen receptors like other steroid receptors share a well-defined domain structure (figure 2).

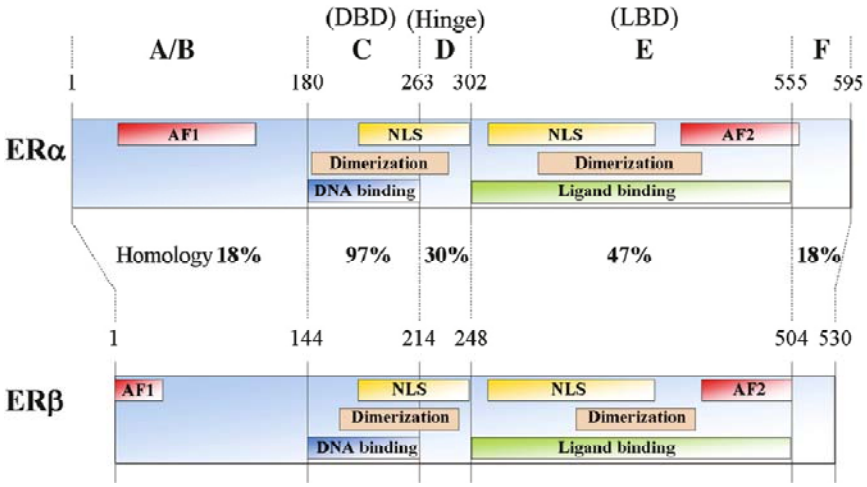


Fig. 2. Schematic representation of human estrogen receptor α and β. Numbers outside each box correspond with amino-acid number. There is also shown a percentage of amino-acid homology (Zilli et al., 2009).

The A/B domain localized at the N-terminus contains the ligand-independent transcriptional activation function 1 (AF1), which allows the receptor to stimulate the transcription. The C domain refers to the DNA-binding domain (DBD) and is also responsible for receptor dimerization. The D domain corresponds to the hinge region. The E domain is involved in the ligand-binding and receptor dimerization and harbours

the ligand-dependent transcriptional activation function 2 (AF2) and the F domain is localized at the C-terminus. Because of the high degree of sequence identity within DNA-binding domain (97%), ER-α and ER-β bind the estrogen responsive element with similar affinity and specifity. The ligand binding domain has a certain degree of amino acid homology and both receptors exhibit similar affinity to endogenous estrogen. On the other hand, there is less than 20% amino-acid identity between A/B domain and C terminal F domain. These differences may contribute to different ER action on the target genes (Zilli et al., 2009). A comparison between AF1 domain of both receptors showed that, under the same conditions, the activity of AF1 of ER-β is minimal while this domain is very active in ER-α on a variety of estrogen responsive elements. These differences between the N-terminal regions could be a possible explanation for the distinct gene regulation and biologic responses (Matthews and Gustafsson, 2003).

Both receptors are expressed in normal breast tissue, but only ER-α is necessary for growth of mammary ducts and normal ductal branching. The experiment with ER-α and ER-β knockout mice demonstrated that ER-α is closely associated with cancer development while ER-β exerts anti-proliferative and pro-differentiative functions. Thus, the loss of ER-β expression is considered to be involved in tumour progression (Matthews and Gustafsson, 2003; Zilli et al., 2009). Despite being discovered nearly ten years after ER-α, ER-β seems to act as major regulator of ER-dependent signalling pathway. In many cases, when ERs are coexpressed, ER-β exhibits an inhibitory action on ER-α mediated gene expression (Matthews and Gustafsson, 2003; Soldati et al., 2009).

1.4 Estrogen signalling pathways

Both receptors act as ligand-activated transcription factors (Moggs and Orphanides, 2001). There are two main signalling pathways (figure 3): the genomic and non-genomic. In the classical genomic pathway in the absence of ligand ER is inactive and bound to heat-shock proteins (Hsp90 and Hsp70). Upon ligand binding ER undergoes structural changes, dissociation from the chaperon proteins and homo-, or heterodimerization and translocation to the nucleus (Ascenzi et al., 2006; Marino et al., 2006; Zilli et al., 2009). In the nucleus dimers bind to a specific DNA sequence, defined as estrogen responsive element (Duffy et al., 2007), in the promoter region of estrogen-

regulated genes which leads to the release of co-repressors and to the recruitment of specific co-activator proteins. These proteins induce up-regulation of genes involved in cell proliferation and survival and down-regulation of genes responsible for anti-proliferative or pro-apoptotic activity (Vasudevan and Pfaff, 2008; Zilli et al., 2009). The above described classical pathway is dependent on the presence of ERE in the promoter of target genes, such as complement 3 and pS-2. The final response to the activation through genomic pathway occurs in hours.

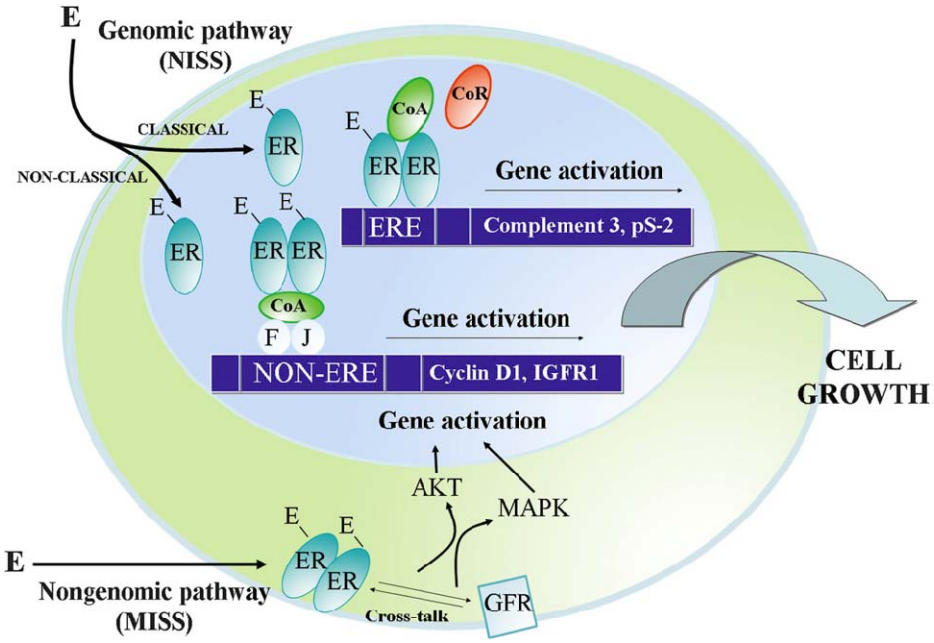


Fig. 3. Genomic and non-genomic estrogen signaling pathways (Zilli et al., 2009).

Estrogens affect also the transcription of genes that do not have ERE sequence. They are regulated by other transcription factors binding to alternative regulatory DNA sequences. ER interacts with transcription factors like activating protein-1 (AP-1), cAMP-responsive element binding protein, NF-κB, Jun/Foz, c-Jun/ATF-2 and Sp-1, stabilize their direct binding to DNA enhancing transcription of genes such as cyklin D1 and insulin-like growth factor receptor-1 (IGFR1). The mechanism is referred as non-classical genomic pathway (Pietras and Marquez-Garban, 2007; Zilli et al., 2009). ER-α and ER-β show similar patterns of genes activation at classical ERE-containing target genes, although ER-β requires higher ligand concentration than ER-α, they exhibit completely different effects at non-classical AP-1 sites after estrogen binding. While ER-α activates, ER-β inhibits transcription. For example expression of cyklin D1

regulated by estrogens via AP-1 sites is stimulated by ER-α but inhibited by ER-β (Zilli et al., 2009).

Some of the ERs are localized in the cell membrane and provide action that is initially independent on gene transcription and thus is referred as non-genomic pathway. Occurring via protein-protein interaction with other transcription factors and signalling complexes that, in turn, mediate gene expression (Pietras and Marquez-Garban, 2007). Non-genomic action of estrogen results in rapid cellular response (milliseconds to minutes) associated with increased calcium levels and activation of intracellular kinase cascades, including phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), protein kinase A and protein kinase C (Chen et al., 2008; Marino et al., 2006; Matthews and Gustafsson, 2003; Vasudevan and Pfaff, 2008). Membrane-associated ER undergoes post-translation modification such as palmitoylation of Cys 447 in the ligand-binding domain of ER-α and is targeted to calveolin-1 localized in lipid rafts. Lipid rafts are cholesterol and sphingolipids-rich domains in cell membrane that provide a matrix for signal transduction. Upon estrogen binding membrane ERs form dimers and interact with adaptor proteins resulting in activation of membrane growth factor receptors as epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2) and insulin-like growth receptor factor (IGFR1). In conclusion, non-genomic activity is influenced by the overall signal transduction pathways and is highly dependent on co-regulatory proteins. ERs and its co-regulators represent targets for phosphorylation by cytoplasmatic kinases leading to activation of nuclear ER-driven transcription (Chen et al., 2008; Katzenellenbogen, 1996; Pietras and Marquez-Garban, 2007; Zilli et al., 2009). Both nuclear and extranuclear estrogen receptors can interact cooperatively with transmembrane growth factor receptor signalling pathways and this cross communication between ERs and GFRs promoters downstream signalling for tumour cell survival, proliferation and also endocrine resistance. Understanding this signalling pathways is necessary for development of new therapies, that will affect this signal transduction (Pietras and Marquez-Garban, 2007).

1.5 Mechanism of estrogen carcinogenesis

Estrogens have long been considered to play a major role in promoting proliferation of normal and neoplastic breast epithelium. Carcinogenesis is a gradual process beginning with genotoxic effects (initiation) that is followed by enhanced cell proliferation (promotion). There are three major mechanisms involved in the carcinogenic effects: stimulation of cell proliferation and gene expression via estrogen receptors, direct genotoxic effects through metabolic oxidative products and via cell membrane-related but ER-independent phosphorylation of target molecules (Chen et al., 2008; Parl et al., 2009; Russo and Russo, 2006). The most widely known mechanism of estrogen carcinogenesis is via binding to its specific nuclear receptor α that exerts a potent stimulus on breast cancer cell proliferation by its direct or indirect action on growth factors production. However some experimental studies showed that in ER-α knockout mice mammary tumours developed (Russo et al., 2003). This observation provides evidence that estrogens may cause breast cancer also through a non-ER-α-mediated mechanism.

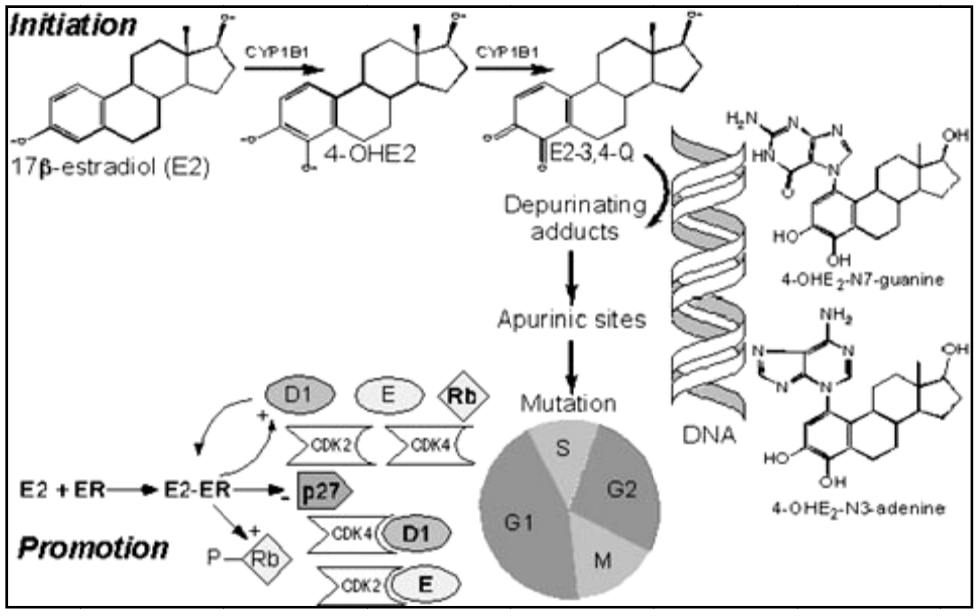


Fig.4: Oxidative estrogen metabolism leading to DNA damage and cell cycle alterations (Parl et al., 2009).

The main estrogen in the breast tissue, 17-β estradiol (E_2), is both a ligand for the estrogen receptor and a substrate for oxidizing enzymes such as CYP1B1. Due to this dual role, E_2 is implicated in the development of breast cancer by stimulating cell

proliferation and by inducing DNA damage through its oxidation products, catechol estrogens. As fig. 4 shows, estradiol is metabolized to catechol estrogens and further to quinones such as 4-hydroxyestradiol (4-OHE_2) and estradiol-3,4-quinone (E_2-3,4-Q) by the above-mentioned enzyme. The highly reactive estrogen quinones form Michael addition products with deoxyribonucleosides. Estrogen quinones and semiquinones undergo redox-cycling resulting in a production of reactive oxygen species causing oxidative DNA damage (Parl et al., 2009).

1.6 Estrogen biosynthesis

There are two sources for steroid hormone formation. Placenta and endocrine glands, which in women include ovaries and adrenals, and peripheral tissues, such as intestine, liver, adipose tissue, kidneys and skin. The first precursor for steroid hormones synthesis is cholesterol (figure 5).

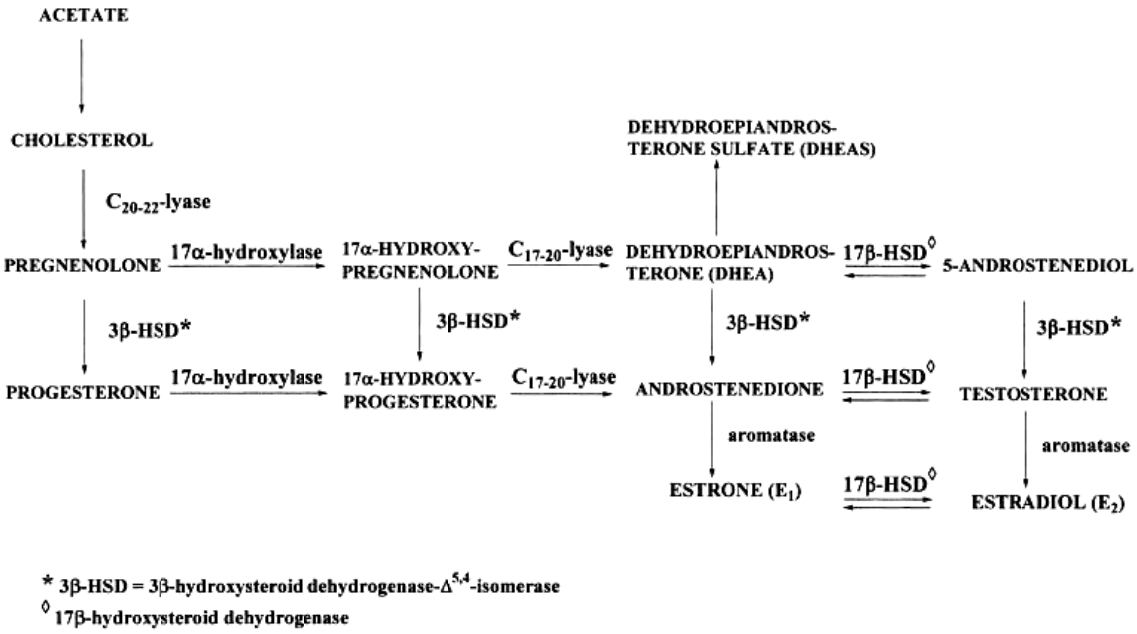


Fig. 5: Biosynthesis of steroid hormones [5].

The first and also rate-limiting reaction in the formation of steroid hormones is the conversion of cholesterol to pregnenolone. Pregnenolone is sometimes referred to as „mother“ steroid because once created it is converted to androgens or estrogens via subsequent reactions catalyzed by steroid dehydrogenases and hydroxylases [5]. The last step of estrogen biosynthesis is catalyzed by aromatase, a member of the cytochrome P450 superfamily, that converts androstenedione and testosterone to estrone (E_1) and estradiol (E_2), respectively (Simpson et al., 2002).

In premenopausal women the main amount of estrogen production comes from ovarian steroidogenesis while in postmenopausal women, whose ovaries decline estrogen formation, the main source of estrogens is extragonadal sites. Estradiol concentrations in breast tissue in premenopausal and postmenopausal women are similar in spite of the decrease of plasma levels in menopause (Castagnetta et al., 1996). Moreover levels of estrogens in women with and without tumours do not differ (Thijssen, 2004). It was also determined, that E_2 concentration present in breast tumour tissues in postmenopausal woman, is 20-fold higher than in the plasma (Simpson and Davis, 2001). The absence of correlation between estrogen levels in circulation and in tissue suggests that there are local factors responsible for the accumulation of relatively high concentrations of estrogens in breast tissue (Castagnetta et al., 1996; Thijssen, 2004). Within peripheral tissues estrogens are not synthesized *de novo*, because extragonadal sites are not able to convert cholesterol to the C_{19} steroids, but are generated locally from circulating C_{19} precursors as androstenedione, dehydroepiandrosterone (DHEA) and DHEA sulfate rising in the adrenal cortex. For this reason circulating levels of androgens play an important role, providing the substrate for estrogen biosynthesis in these sites (Simpson et al., 2002; Simpson and Davis, 2001). There are two important steroidogenic reactions occurring in peripheral tissues. The first is the conversion of androgens to estrogens in adipose tissue that has high activity of aromatase and after menopause becomes the main source of estrogen. The second is transformation of testosterone to dihydrotestosterone (DHT) in the skin [5]. It seems that androgens might be more important for maintaining local level of E_2 than circulating estrogens.

1.7 Aromatase

Estrogen biosynthesis is catalyzed by aromatase, a microsomal enzyme, that belongs to the cytochrome P450 superfamily and is encoded by the CYP19 gene. The cytochrome P450 multicomponent mono-oxygenase system cooperates with its redox partner NADPH-cytochrome P450 reductase (CPR) that contains flavins, FAD and FMN, and catalyze transfer of electrons from NADPH to aromatase. Estrogen synthesis is a three-step process (figure 6). Each step requires 1 mol of O_2 and 1 mol of NADPH. Upon receiving electrons from CPR aromatase converts androgens (C_{19}) to estrogens (C_{18}) by three successive hydroxylations (Hong et al., 2009). The first and second hydroxylations occur at the 19-methyl group of androgen and the third step involves cleavage of the bond between C_{10} and C_{19} and the aromatization of the steroid A-ring. The aromatization step is still poorly understood although it has been studied for more than 35 years (Ghosh et al., 2009).

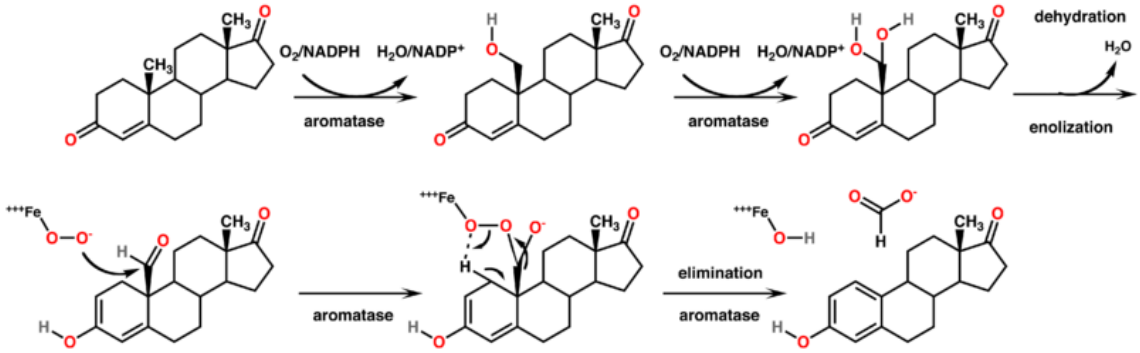


Fig.6: Mechanism of last step in estrogen biosynthesis catalyzed by aromatase [6].

Aromatase expression is under the control of tissue specific promoters regulating alternative splicing mechanism in various sites. In ovaries expression is primarily mediated by promoter II, in placenta by promoter I.1 and in adipose tissue and skin by promoter I.4. Tumours produce factors, that stimulate local aromatase expression, that is associated with aromatase promoter switching from glucocorticoid-stimulated promoter I.4 in normal tissue to cAMP-stimulated promoters I.3 and II in cancerous tissues (Chen, 1998; Simpson et al., 2002).

Since estrogens play an important role in breast cancer development there has been a long-term interest in the structure-function relationship and many attempts to elucidate aromatase structure have been done. In a recent work, Ghosh et al. (2009) revealed the crystal structure of human aromatase (figure 7). These findings clarified the molecular mechanism of androgens conversion to estrogens and are very important for the development of new aromatase inhibitors for prevention or/and treatment of hormone-dependent breast cancer (Hong et al., 2009).

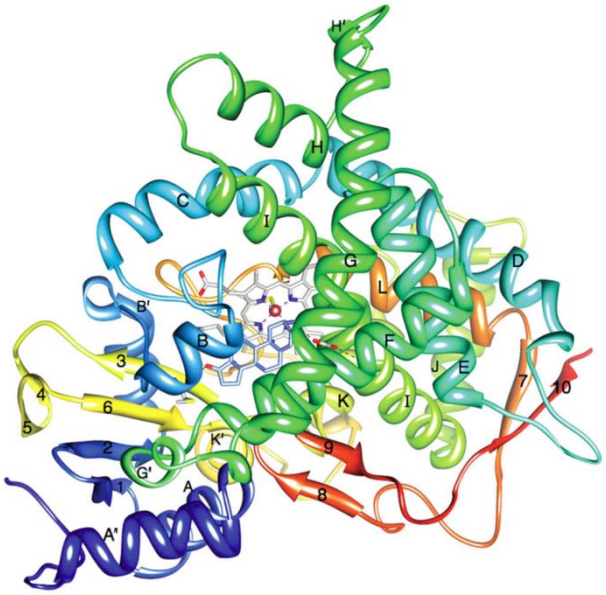


Fig. 7: The structure of human aromatase P450 enzyme (Ghosh et al., 2009).

1.8 Breast cancer therapies

Breast cancer can be prevented by surgical (bilateral mastectomy or oophorectomy) or by endocrine approaches (tamoxifen or raloxifene). There is a high percentage of estrogen receptor positive breast cancer and for this reason there are lot of efforts to develop drugs, that block the estrogen receptors (anti-estrogens like tamoxifen) or prevent the synthesis of estrogens (aromatase inhibitors).

Tamoxifen, the pioneering synthetic selective estrogen modulator (SERM), was the first drug developed for treatment of estrogen receptor positive breast cancer. It also became the first drug, that was approved by the United States Food and Drug Administration for the prevention in high risk women (Oseni et al., 2008). Tamoxifen is

a synthetic non-steroidal agent that binds to the both ERs with equivalent affinity and induces conformation changes. These changes result in different amino acids exposure on the receptor and also promote the binding of co-regulators, which determine the biologic activity of the SERM at the affected tissues (Johnston and Dowsett, 2003). In conclusion, SERMs show partial agonist and also antagonist activities depending on the tissue type. For example tamoxifen is an antagonist in breast tissue, but it acts as partial agonist on the endometrium (Dutta and Pant, 2008). Except increased risk of endometrial cancer there are other side effects including formation of blood clots and stroke, decrease of bone density in pre-menopausal women and fertility issues along with hair and nail thinning (Johnston and Dowsett, 2003). Despite of these side effects tamoxifen has been the gold standard for treatment of ER-positive breast cancer for many years and has saved thousands of women´s lives. Another SERM, Raloxifen, which was primarily used for osteoporosis treatment, has also shown significant reduction in breast cancer development even with better side effect profile than tamoxifen. Besides SERMs, there is a group of selective estrogen receptor degradation agents (SERDs) such as fulvestrant. They directly target estrogen receptor and cause its degradation.

Other alternative approach is treatment with aromatase inhibitors (AIs). However, this approach is suitable only for post-menopausal women. AIs are categorized into two types, steroidal and non-steroidal depending on their structure and mechanism of action. Steroidal aromatase inhibitors are analogues to androgens structure and compete with aromatase substrate for binding to the active site. Non-steroidal inhibitors interact directly with heme prosthetic group of aromatase (Dutta and Pant, 2008; Chen, 1998). The first aromatase inhibitor was aminoglutethimide, but lack of specifity and presence of side effects including liver toxicity, cortisol production inhibition and hypothyroidism led to the withdrawal of its use (Dutta and Pant, 2008) and development of second generation aromatase inhibitors like Formestane (4-hydroxyandrostenedione; 4-OHA) and Fadrozole (non-steroidal counterpart of 4-OHA). Formestane, a steroidal inhibitor, showed high efficacy against ER-positive breast tumours, but it was ineffective in patients with no prior endocrine therapy response. Moreover, formestane has to be administrated by muscular injections. Second generation did not receive a lot of attention, because third generation quickly followed. Third generation include the non-steroidal inhibitors anastrazole and letrozole, and the

steroidal exemestane. This class of therapeutics were developed to fight against breast cancer cases that do not respond to tamoxifen treatment. They have minimal harmful effects and are highly selective in comparison with previous aromatase inhibitors (Dutta and Pant, 2008).

Despite of significant success in improving the survival rates among breast cancer patients there is a risk of developing resistance to the available therapeutic approaches. Further investigations of new compounds with aromatase inhibitors features are required for ongoing combat of ER-positive breast cancer.

1.9 Flavonoids

Flavonoids, derived particularly from soya, have started to be examined because of a low incidence of breast cancer that has been noticed in countries with high soy intake. This observation has led to the extensive research of a potential role for phytestrogens in preventing breast cancer development (Oseni et al., 2008). Initial excitement over the putative anticancer features of soya was proved in 1966 by Folman and Pope (1966), who were the first to demonstrate anti-estrogenic effects of isoflavones. Later it was discovered that isoflavone genistein can act as a specific inhibitor of tyrosine protein kinase (PTK), which is frequently over-expressed in cancer cells. Nowadays genistein is thought to be able to inhibit the activity of many enzymes including topoisomerase II, PTK (Peterson and Barnes, 1996), 17β-hydroxysteroid dehydrogenase (Brooks and Thompson, 2005) and aromatase (Messina and Loprinzi, 2001).

Flavonoids can be categorized into flavones, flavanols, isoflavones, flavonols, flavanones, and flavanonols according to the saturation level and opening of the central pyran ring. The most common form of flavonoids is isoflavones that are found in a variety of plants, but the highest dietary source is soya. There are two main isoflavones, genistein and daidzein, present in soy, in their β-D-glycosides form the genistin and dadzin. The intestinal bacteria contain glucosidases that hydrolyze glycosidic bonds and produce aglycone forms of isoflavones that are biologically active (Oseni et al., 2008).

Soya foods, because of a number of health benefits, have become very popular among non-Asians. In Japan and China adult people consume between 25-50 mg isoflavones per day. In contrast, daily soya intake is quite low in the Western countries- typically less than 3 mg/day, however among vegetarians and health-conscious individuals amounts of soya foods are much higher (Messina and Wood, 2008). The modest consumption of isoflavones (less than 100 mg/day) showed favourable effects in humans, such as enhancing arterial compliance and reducing urinary levels of 5-hydroxymethyl-2-deoxyuridine, which is a marker of oxidative DNA damage (Messina and Loprinzi, 2001).

Flavonoids are a group of substances that have structural similarity to estradiol. The presence of the phenolic ring allows them to bind to estrogen receptors and induce a similar response. Due to this feature they are called phytestrogens. It has been discovered that they have estrogen agonistic and antagonistic effects (Oseni et al., 2008) and for this reason, they are sometimes classified as selective estrogen receptor modulators (SERMs) (Messina et al., 2006). In comparison with SERMs that in general can bind to ER-α or ER-β with similar affinity, phytestrogens appear to bind to ER-β with greater preference. This action may explain how phytestrogens can be protective against breast cancer, because binding to ER-β leads to mammary cell growth inhibition as well as inhibition of ER-α stimulatory effect. Phytestrogens are considered to be weakly estrogenic, having 10^{-5}-10^{-2} less activity per mole compared with 17β-estradiol. However, in some *in vitro* studies isoflavones exhibited estrogenic effects even greater than estradiol (Duffy et al., 2007). *In vitro* examinations of estrogenic and anti-estrogenic potential of isoflavones have shown that genistein acts primarily as an agonist of ER-β and that the different transcriptional actions of estrogens and isoflavones result not only from distinct binding affinities but also from different ability to recruit co-regulators and trigger transcription function of ERs.

As mentioned above, isoflavones have been shown to inhibit several important enzymes involved in estrogen metabolism. Namely aromatase, a cytochrome P_{450} enzyme, responsible for the production of estrogens from androgens, 17β-hydroxysteroid dehydrogenase convert estrone (E_1) to a more potent compound, estradiol (E_2). Low micromolar concentrations of isoflavones inhibit 3β-hydroxysteroid dehydrogenase converting dehydroepiandrosterone into androstenedione, that can be

subsequently converted to estrone (Messina and Loprinzi, 2001). Isoflavones also inhibit several types of cancer cells *in vitro* including ER-dependent as well as ER-independent cancer cell lines. Further, isoflavones exert anti-inflamatory, anti-atherogenic, anti-allergic and antioxidant activity and inhibit angiogenesis. Isoflavones are also considered as an alternative to hormone therapy in post-menopausal women (Helferich et al., 2008).

A variety of health benefits have been attributed to soya consumption, but there is also a concern arisen over their estrogenic properties, that may increase the risk of recurrence or stimulate growth of pre-existing tumours. There are some studies showing, that genistein *in vitro* stimulates the growth of ER-positive mammary cancer cells. *In vivo*, ovariectomized athymic mice dietary feeded with genistein exerted the stimulation of growth of existing ER-positive breast tumours (Messina et al., 2006). The role of phytestrogens in breast cancer prevention remains unclear. The impact of soya intake on breast cancer patients and woman at high risk of this disease needs to be better understood.

2. AIM OF THE STUDY

There are several terapeutic approaches for treatment of ER-positive breast cancer. The inhibition of the aromatase enzyme is one of them. Aromatase is responsible for catalyzing the last step of estrogen biosynthesis – a conversion of androstenedione and testosterone to estrone and estradiol, respectively. On the other hand, there is a strong evidence that isoflavones have the ability to bind estrogen receptors and are important inhibitors of carcinogenesis. Moreover, some studies have shown that these compouns inhibit cell proliferation of different cancer cell lines. However, the mechanism by which ginestein and dadzein modulates growth of breast cells and prevents breast cancer are not fully understood. In that way, the aim of this study was to investigate if the soya extract biotransformed by fungi *Aspergillus awamori,* which contains ginestein and daidzein, was able to inhibit aromatase activity, in placental microsomes and in MCF-7aro cells, an estrogen dependent human breast cancer cell line stably transfected with the aromatase gene. This cell line will also be used to evaluate the potential effects of the biotransformed soya extract on breast cancer cell proliferation.

3. MATERIALS AND METHODS

3.1 Materials

The soya extract biotransformed by fungi *Aspergillus awamori* was obtained from the laboratory of Prof. Maria José Vieira Fonseca and Prof. Maria Regine Torqueti of Faculty of Pharmacy of Ribeirão Preto, University of São Paulo in Brazil. Eagle´s essential minimum medium (MEM), phenol red free medium, fetal bovine serum, antibiotics penicilin-streptomycin-amphotericin B, Geneticin (G418), trypsin and L-glutamin were purchased from Gibco Invitrogen Co. (Parsley, Scotland, UK). Formestan (4-OHA), testosterone, reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), ethylenediamine tetraacetic acid (EDTA), trypan blue, dimethylsulphoxide (DMSO), dextran, charcoal and sodium pyruvate were obtained from Sigma-Aldrich Co. (Saint Louis, USA). Tritiated androstenedione was purchased from Perkin-Elmer (Boston, USA). Scintillating liquid cocktail was from ICN Radiochemicals (Irvine, USA), dye reagent for Bio-Rad protein assay was supplied by Bio-Rad (München, Germany). Tritiated thymidine was from Amersham (Amersham International, Amersham, UK). MCF-7aro, the ER-positive human breast cancer cells stable transfected with human placental aromatase gene and selected with geneticin were kindly supplied by Prof. Shiuan Chen from the Beckman Research Institute (City of Hope, Duarte, CA, USA).

3.2 Methods

3.2.1 Preparation of charcoal pellets

To prepare charcoal pellets a suspension of 5% charcoal, 0,5% dextran in PBS was prepared and 1 ml of this mixture was put into each microcentrifuge tubes and centrifuged at 14 000 g for 10 min. The supernatant was removed and the charcoal pellets were dried. For in-cell aromatase assay the volume was reduced to 500 µl of charcoal-dextran mixture.

3.2.2 Preparation of charcoal dextran heat-inactivated FBS

The fetal bovine serum was inactivated at 56 ºC for 1 h. Then, 500 ml of heat-inactivated FBS was treated with 8 g activated charcoal at room temperature during 24 h, 5-6 times centrifuged at 4000 g/15 min and passed through 0,22 μm filter to separate the charcoal from the serum.

3.2.3 Preparation of placental microsomes

Aromatase inhibition studies were performed using human placental microsomes that contain a high level of aromatase. Placental microsomes were obtained as described by Yoshida and Osawa (1991), with some modifications. Human placentas delivered from a local hospital were placed in cold 67 mM potassium phosphate buffer (pH 7,4) that contained 1% KCl. All procedures were performed at 0-5 ºC. The cotyledon tissue was separated and homogenized in a Polytron homogenizer with 67 mM KH_2PO_4 (pH 7,4) containing 0,5 mM dithiotreitol (DTT,1:1,w/v) and 0,25 M sucrose. The homogenate underwent a differential centrifugation. First it was centrifuged at 5000 g for 30 min. Then the supernatant was taken and centrifuged twice at 20 000 g for 30 min and at 54 000 g for 45 min to obtain the microsomal pellet. The microsomes were washed and resuspended in 67 mM potassium phosphate buffer (pH 7,4), which contained 0,25 M sucrose, 20% glycerol and 0,5 mM DTT and stored at -80 ºC. The protein concentration was determined by Bradford protein assay using bovine serum albumin as a standard.

3.2.4 Aromatase assay procedure

Aromatase activity was measured according to Thompson and Siiteri (1974). This procedure is based on the evaluation of the amount of tritiated water released from radiolabeled androstenedione, during the aromatization reaction. The soja extract was dissolved in DMSO and diluted in 67 mM potassium phosphate buffer (pH 7,4). The reaction mixture, of total volume 1 ml, contained 20 μg of protein of the microsomes, different dilutions of the soya extract (corresponding to the concentration 1,97 μM, 3,9 μM and 5,9 μM of Daidzein and the concentration 1,48 μM, 2,9 μM and 4,44 μM of Genistein), 40 nM of [1β-^{3}H] androstenedione and an appropriate amount of KH_2PO_4 to complete the final volume of 1 ml. As a positive control 0,5 μM formestane was

used. The blank contained the same substances except the extract. The reaction was initiated by the addition of 150 μM reduced nicotinamide adenine dinucleotide phosphate (NADPH) and incubated in a shaking water bath at 37 ºC, for 15 minutes. To stop the reaction 250 μl of 20% TCA was added into each tube. Then the mixture was transferred to the eppendorf tubes with pre-made charcoal-dextran pellet, vortexed and incubated at room temperature, for 1 hour. After the centrifugation at 14000 g, for 10 minutes the supernatants were transferred into new charcoal-dextran pellets, vortexed, incubated for other 10 minutes and centrifuged under the same conditions. The supernatant from each tube was transferred to empty eppendorf tubes and centrifuged to avoid the presence of the charcoal, which could cause some interference. Then, 600 μl aliquot of supernatant containing the tritiated-water product was mixed with 3 ml of a liquid scintillating cocktail and measured in a liquid scintillation counter. Experiments were performed in triplicate.

3.2.5 Cell cultures

MCF-7aro is a human breast ER-positive cancer cell line which was stably transfected with aromatase gene. To maintain the cell line in culture, cells were seeded in 75 cm^2 plastic flasks with a standard MEM medium containing Earle´s salt, 2 mM L-glutamine, 1mM sodium-pyruvate, 1% penicillin-streptomycin-amphotericin B, 70 ng/ml geneticin (G418) and 10% heat-inactivated FBS and incubated at 37 ºC with 5% CO_2 atmosphere. The medium was changed every 3 days. When the cells reached 80-90% of confluence, they were washed with PBS and incubated with 2 ml of 0,25 % trypsin with 1mM EDTA, for 2 min, at 37 ºC. The flask was rinsed with medium containing FBS to stop the action of trypsin. The detached cells were taken out and centrifuged at 400 g, at 4 ºC, for 6 min. The pellet was resuspended in MEM and seeded into new flasks. In order to count the cells after trypsinization an aliquot was mixed with the vital dye Trypan Blue 0,1% (1:1) and the viable cells (a viable cell does not stain with trypan blue) were counted under the microscope using a Neubauer chamber.

3.2.6 In-cell aromatase activity

Aromatase converts androgens to estrogens through three hydroxylations and during this reaction product is released from androst-4-ene-3,17-dione. Aromatase activity was determined according to Thompson and Siiteri (1974) with modifications.

MCF-7aro cells ($1x10^6$ cells/well) were cultured in 24-well plates in 500 µl Eagles's minimum essential medium (MEM) with 10% heat-inactivated fetal bovine serum (FBS). When the cells were confluent, they were washed with PBS and cultured in 300 µl serum-free medium (MEM) with 50 nM [1β-^{3}H] androstenendione and 500 nM progesterone (to supress 5α-reductase, which also metabolizes androstenedione), and soya extract containing 5,7 µM to 57 µM of Genistein and 7,6 µM to 76,0 µM of Daidzein. The cells were incubated for 1 hour at room temperature. The reaction was stopped by addition of 100 µl TCA 20%. The content of each well was transferred to pre-made charcoal-dextran pellets, incubated for 1 hour at room temperature and centrifuged at 14 000 g for 10 minutes. Then, the supernatant was taken to other charcoal-dextran pellets and incubated for 10 minutes. After centrifugation 200 µl of the supernatant were mixed with 3 ml of liquid scintillation cocktail and the percentage of inhibition was determined in scintillation counter (LS-6500, Beckman Coulter). All tests were carried out in triplicate. As a positive control formestane at 1 µM was used. The blank contained cells in serum-free medium without soya extract. In order to determine the protein content, that corresponds to the obtained aromatase inhibition, cells were lysed with 1 ml of 0,5 M NaOH, overnight at room temperature under constant agitation, and protein concentration was evaluated by the Bio-Rad Protein Assay.

3.2.7 Bio-Rad Protein Assay

The procedure is based on the method of Bradford with the binding of Coomassie Blue G-250 to proteins. The unknown concentration of protein was determined by comparison to a standard curve using bovine serum albumin (BSA) as a standard. The BSA was diluted with distilled water to obtain the concentration of 0,1 mg/ml and 10, 20, 30, 40 and 50 µl of this solution were pipeted into a 96-well plate and H_2O was added to total a volume of 160 µl. The sample of unknown concentration

was also diluted with H_2O. The Bradford Reagent (40 µl) was added and the absorbance was measured at 595 nm. All determinations were done in triplicate.

3.2.8 Thymidine incorporation assay

To evaluate DNA synthesis the thymidine incorporation assay was used. In order to prevent estrogenic effects of FBS and phenol red (Wesierska-Gadek et al., 2007) the cells were cultured three days before the assay in phenol-red free medium containing 1mM sodium-pyruvate, 2mM L-glutamine, 1% penicillin-streptomycin-amphotericin B and 5% charcoal-stripped heat-inactivated fetal bovine serum (CFBS). Cells were seeded in 96-well plate in a density of 2,5 x 10^4 cells/ ml, in 200 µl of the above-mentioned medium with 1 nM testosterone and different concentrations of soya extract (corresponding to 1,5 µM, 750 nM, 325 nM and 50 nM of Genistein and 1,92 µM, 958,5 nM, 415 nM and 64 nM of Daidzein) and incubated for 48 hours at 37°C. The [^{3}H]-thymidine was added to each well 8 hours before the assay. To lyse the cells a cycle of freezing/unfreezing was carried out. Then, every liquid were sucked out using the semi-automated cell harvester (Scatron Instruments, Norway) and the DNA was retained in a special filter. The filter circles were put into the tubes with 1 ml of scintillation liquid to determine the [^{3}H]-thymidine incorporation in DNA, using scintillation counter (LS 6500, Beckman Coulter). The blank did not contain soya extract only the appropriate medium. All the experiments were done in triplicate.

3.2.9 Giemsa staining

For the cell morphology study the Giemsa staining was used. Cells were seeded in 8 well chamber slides in a density 6 x 10^4 cells/ ml in 400 µl of phenol-red free medium with 1 nM testosterone and incubated with soya extract for 48 hours at 37°C under the same conditions as referred for the thymidine incorporation assay. Then, the incubation medium was removed, cells were washed with PBS, fixed with methanol (400 µl into each well) for 30 minutes, at room temperature. After washing with PBS the cells were incubated with Giemsa stain for 30 minutes at room temperature, then, washed with tap water and mounted in DPX (a mixture of Distyrene, a plasticizer, and xylene).

3.2.10 Preparation of the soya extract samples

The biotransformed soya extract, prepared by Prof. Maria Regine Torqueti from the Faculty of Pharmacy of Ribeirão Preto, University of São Paulo, Brazil, contained a mixture of two isoflavones, genistein and daidzein in different concentrations. The sample was obtained as an extract, which was dissolved differently depending on the procedure:

- For aromatase assay in microsomes the extract was dissolved in DMSO (100 µl) and PBS (900 µl) to obtain the *mother solution* of 116 mg/ml that was subsequently diluted to 5 mg/ml solution containing 5 ug/µl of daidzein and 4 µg/µl of genistein.
- For in-cell aromatase assay the extract was dissolved in DMSO (500 µl) and serum-free medium (500 µl) to obtain the *mother solution* of 116 mg/ml that was subsequently diluted (1:2) to 58 mg/ml solution containing 58 ug/µl of daidzein and 46,4 µg/µl of genistein.
- for thymidine incorporation assay the extract was dissolved in DMSO (500 µl) and phenol-red free medium with charcoal-stripped heat-inactivated fetal bovine serum (500 µl) to obtain the *mother solution* of 116 mg/ml that was subsequently diluted (1:2,9) to 40 mg/ml solution containing 40 µg/µl of daidzein and 32 µg/µl of genistein.

All the stock solutions were stored in -20 ºC. For further dilutions an appropriate medium or buffer was used. The non-biotransformed soya extract was obtained as a mixture of genistein and daidzein in a concentration of 5 µg/ml of daidzein and 4,81 µg/ml of genistein. The preparation of the extract was as for the biotransformed soya extract.

4. RESULTS

4.1 Evaluation of the aromatase activity in microsomes

The biotransformed soya extract was firstly screened for putative aromatase inhibition properties. For this reason, we evaluated the aromatase activity, in placental microsomes, in the presence of 300 µl, 200 µl and 100 µl of the extract with 5 mg/ml of total concentration of isoflavones. These volumes corresponded to the concentrations of genistein and daidzein shown in Table 1. For comparison, there are also shown the concentrations of the isoflavones in non-transformed samples. The results suggest that our extract is able to inhibit aromatase activity in a dose-dependent manner (figure 8). For 10,34 µM, 6,8 µM and 3,45 µM of total concentration of isoflavones in the biotransformed soya extract we obtained 87,3%, 69,8% and 40,4% of aromatase inhibition, respectively.

Table 1. Concentration of isoflavones in the soya extracts (biotransformed and non-biotransformed)

Volume of biotransformed soya extract	**Concentration of Genistein(G) and Daidzein (D)**
100 µl	1,48 µM of G + 1,97 µM of D (3,45 µM)
200 µl	2,9 µM of G + 3,9 µM of D (6,8 µM)
300 µl	4,44 µM of G + 5,9 µM of D (10,34 µM)
Volume of non-biotransformed soya extract	**Concentration of Genistein (G) and Daidzein (D)**
100 µl	1,78 µM of G + 1,97 µM of D (3,75 µM)
200 µl	3,56 µM of G + 3,9 µM of D (7,46 µM)
300 µl	5,3 µM of G + 5,9 µM of D (11,2 µM)

To find out if the biotransformation induced by the fungi had any influence in the anti-aromatase activity, we carried out an aromatase assay using a sample of soya extracts without fungi under the same conditions as for the biotransformed one. The results are only preliminary, though it was obtained 90,13%, 86,62% and 69,88% of aromatase inhibition, respectively for 11,2 µM, 7,46 µM and 3,75 µM of total concentration of isoflavones in the soya extract (figure 9).

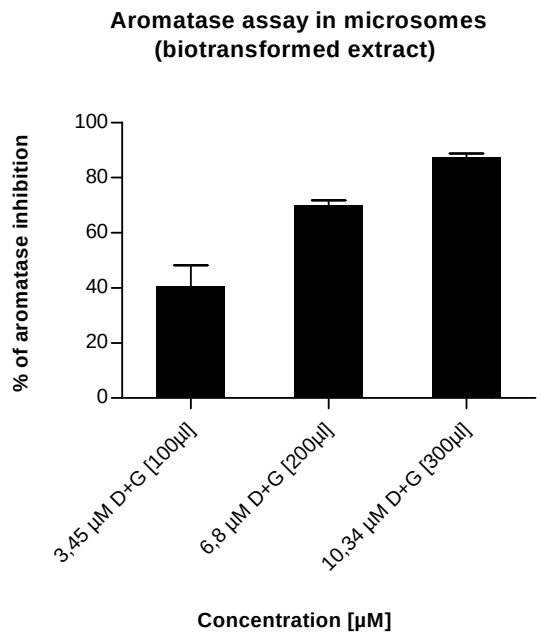


Figure 8.Dose-dependent inhibition of aromatase activity in microsomes. The results are presented as mean of three independent experiments each carried out in triplicate.

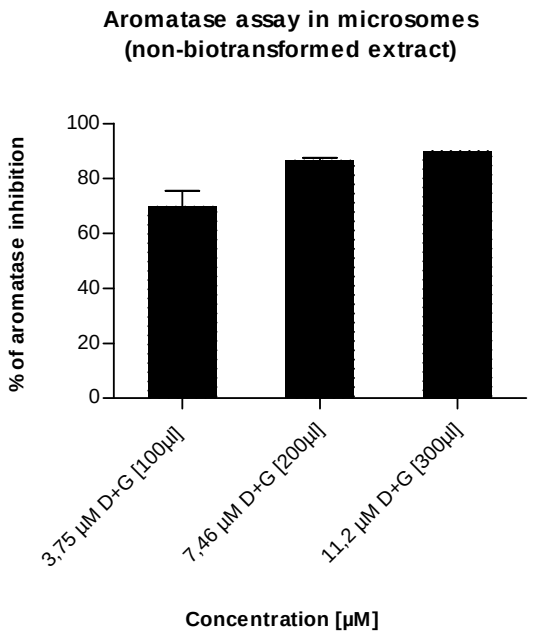


Figure 9. Dose-dependent inhibition of aromatase activity in microsomes. This graph represents only preliminary results of aromatase inhibition in non-biotransformed soya extract.

Our results suggested that the non-transformed soya extract was more potent to inhibit aromatase activity (figure 9). This higher aromatase inhibition is probably due to a little difference in the total content of isoflavones in both extracts. The non-transformed sample has a higher concentration of genistein. To compare concentration differences see Table 1. The total concentration of isoflavones that was able to induce 50% of aromatase inhibition was of 4,5 µM (figure 10).

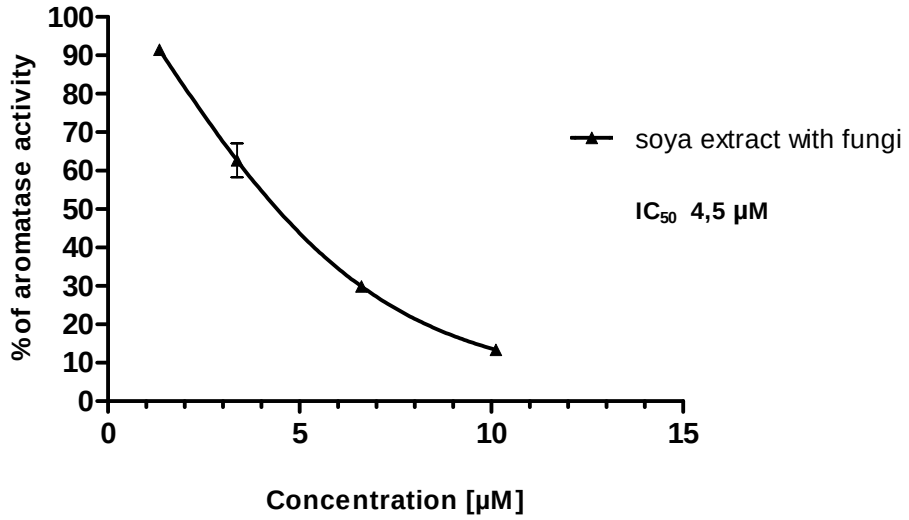


Figure 10. The effect of soya extract on aromatase activity. For aromatase activity and IC_{50} determination were used 20 µg of placental microsomes, 40 nM of tritiated androstenedione and different concentrations of isoflavones presented as total sum of Genistein and Daidzein.

4.2 Evaluation of anti-aromatase activity in MCF-7aro cell line

The placental microsomal assay showed that the isoflavones contained in the biotransformed soya extract were able to induce aromatase inhibition. Our further interest was to find out if the biotransformed soya extracts inhibited aromatase activity in MCF-7aro cells. This cell line is an ER-positive breast cancer cell line, stably transfected with the aromatase gene, which is consider being a good model to study compounds with anti-aromatase activity in breast cancer management.

The in-cell aromatase assay was performed with 10µl, 20 µl, 40 µl, 80 µl and 100 µl of soya extract that corresponded to 5,73 µM G + 7,6 µM D, 11,5 µM G + 15,2 µM D, 22,9 µM G + 30,4 µM D, 45,9 µM G +60,8 µM D and 57,3 µM G + 76,0 µM D, respectively. The percentage of aromatase inhibition obtained was approximately 25% for the concentration of 13,35 µM and of 85% for the concentration of 53,3 µM, while for the higher concentrations the inhibition was almost complete. Figure 11 shows the percentage of aromatase activity per µg of protein, presented as total content of both isoflavones. The concentration of total isoflavones in the extract that induced 50% of aromatase inhibition was 21 µM.

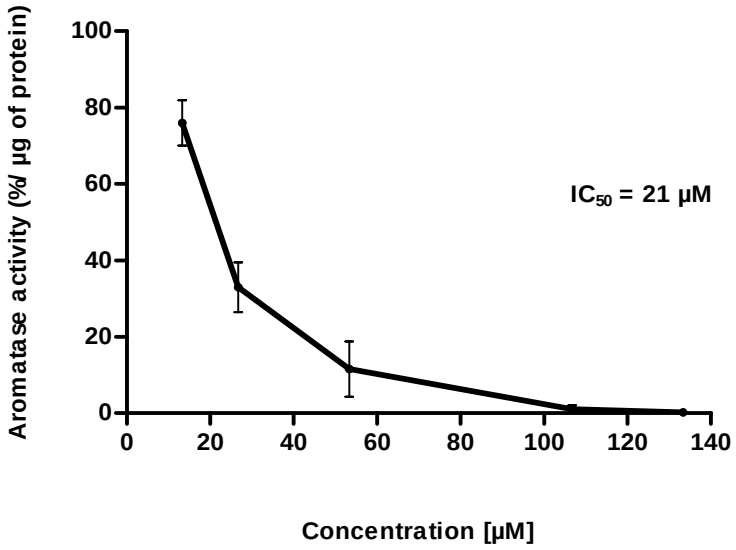


Figure 11. Dose-dependent inhibition of aromatase activity in MCF-7aro cells. Concentration is shown as a total sum of genistein and daidzein contained in biotransformed soya extract. The results are presented as mean ± SD of three independent experiments each carried out in triplicate.

4.3 Morphological studies

To examine if biotransformed soya extract induced any morphological alterations in MCF-7aro cells, phase contrast microscopy and Giemsa staining were performed (figure 12). The untreated control cells did not show any morphological changes during 48 hours of incubation. After 48 hr of treatment with the lower concentration (50 nM of Genistein plus 64 nM of Daidzein), the cells did not show morphological alterations, though, a few non-adherent round cells and membrane blebbing, a characteristic for apoptotic cell death, was detected. For the highest concentration of isoflavones (1,5 µM of Genistein plus 1,92 µM of Daidzein) a massive cell death was observed. Almost all the cells were detached and there was abundant cell debris.

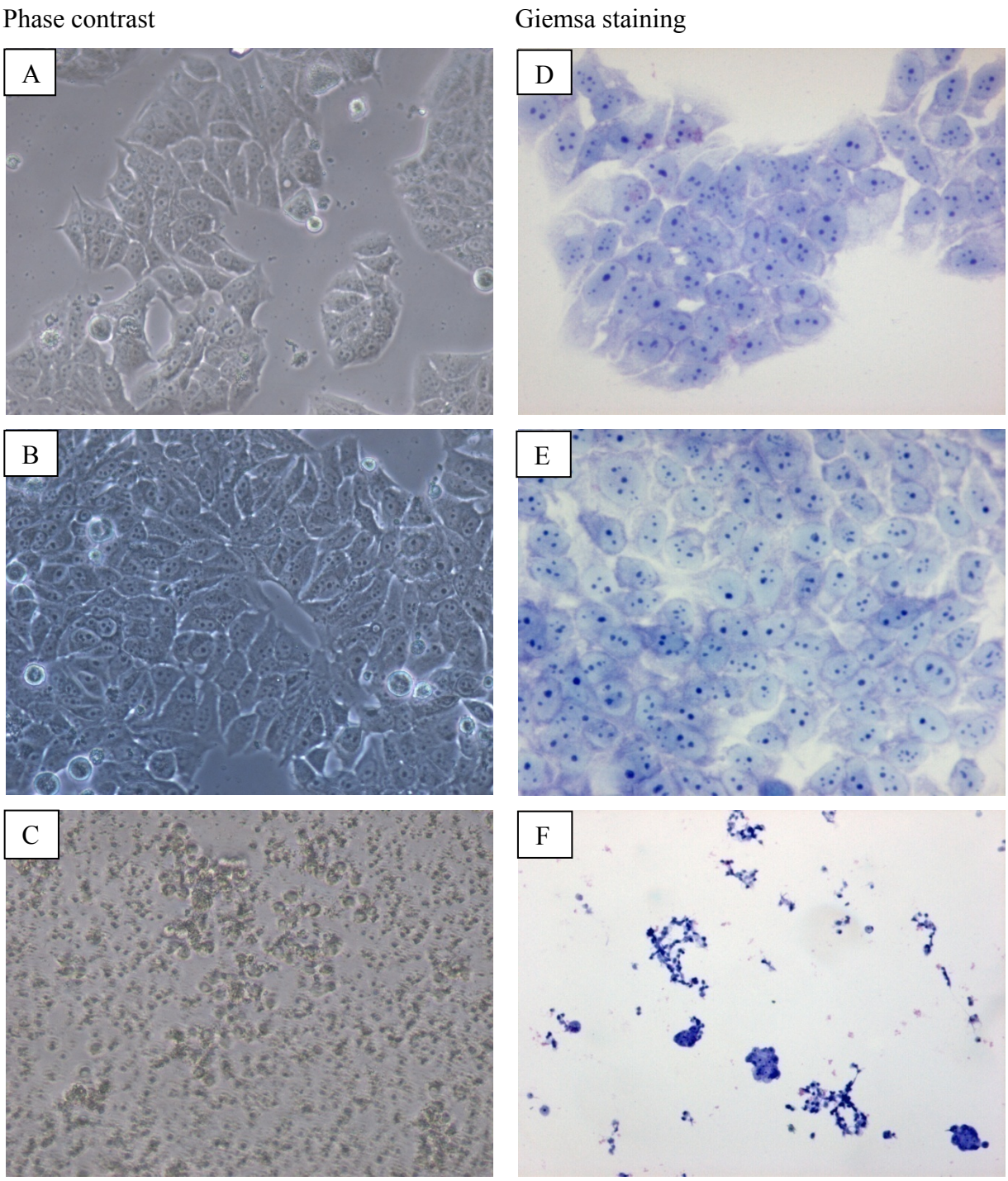


Figure 12. Morphological alterations of MCF-7aro cells treated for 48 hr with biotransformed soya extract in different concentrations. In pictures A-C are shown phase contrast microscopies while in pictures D-F are presented Giemsa staining. Untreated control cells in phase contrast (A) and stained with Giemsa (D) (magnification 400x) without any morphological changes. The images B and E (magnification 400x) represent cell treatment with low (0,11 μM) concentrations. Images C and F (magnification 100x) show a massive reduction in cell viability in response to the treatment with higher (3,42 μM) concentrations of genistein and daidzein.

4.4 Evaluation of cell proliferation

In order to investigate the direct effects of biotransformed soya extract on the proliferation of MCF-7aro cells, thymidine incorporation assay was performed. Cells were treated with different concentrations of isoflavones, corresponding to 3,42 μM (1,5 μM of genistein + 1,92 μM of daidzein), 1,71 μM (0,75 μM G + 0,9585 μM D), 0,74 μM (0,325 μM G + 0,415 μM D) and 0,11 μM (0,05 μM G + 0,064 μM D) as shown in figure 13. In the concentration of 0,11 μM (0,050 μM of Genistein plus 0,064 μM of Daidzein) only a slight decrease in DNA synthesis was observed, while 0,74 μM (0,325 μM of Genistein plus 0,415 μM of Daidzein) caused a reduction of 20% in cell proliferation. Above this latter concentration a dramatic decrease in DNA synthesis was detected.

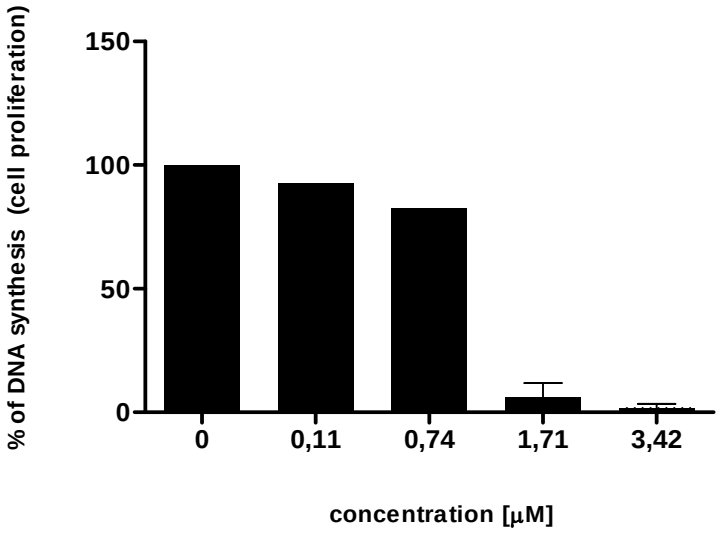


Figure 13. Effect of the soya extract on the rate of DNA synthesis. MCF-7aro cells cultured in steroid-free medium without red phenol and treated with four different concentrations of biotransformed soya extract in medium containing 1 nM of testosterone. Cells treated only with testosterone represented maximum cell proliferation and were considered as a control. Results are shown as percentage of DNA synthesis and concentration of the soya extract as a total of genistein and daidzein.

5. DISCUSSION AND CONCLUSIONS

Epidemiological studies have shown that high soya intake is associated with a reduced breast cancer risk. Though, it has also been shown that the isoflavones present in soya exhibit estrogen-like properties, inducing proliferation and estrogenic markers in MCF-7 cells. In order to clarify the mechanisms by which ginestein and dadzein, the predominant isoflavones present in soya, prevents breast cancer we examined the *in vitro* effects of soya extract on aromatase activity in human placental microsomes and in an ER-positive breast cancer cell line (MCF-7aro).

We found that the biotransformed soya with the fungi *Aspergillus awamori* and non-transformed soya extract effectively inhibit aromatase activity in human placental microsomes in a dose-dependent manner. In addition, our results also showed that, in placental microsomes, the biotransformation of soya did not alter the capacity to induce aromatase inhibition. Preliminary results with the non-transformed sample showed greater aromatase inhibition. One possible explanation to this result may be the higher concentration of ginestein in this extract, which resulted in a slight increase in the total concentration of the isoflavones present in the extract. These findings are not so consistent with Le Bail et al. (2000), who found that isoflavonoids with the phenolic B ring in the 3 position on the pyran ring (genistein and daidzein), are not potent aromatase inhibitors, being a stronger 3β-hydroxysteroid dehydrogenase and/or 17β-hydroxysteroid dehydrogenase inhibitors. In contrast, flavonoids with B ring at position 2 (7-hydroxyflavone, chrysin, apigenin) are more potent aromatase inhibitor. Moreover, Brooks and Thompson (2005) also reported, that genistein and daidzein has little or no ability to inhibit aromatase activity in placental microsomes, but modulate activity of 17β-hydroxysteroid dehydrogenase. Other authors studied various flavonoids, four flavones (for example chrysin), two isoflavones (genistein and biochain A), flavanone (naringenin) and napthoflavone and concluded that flavones are much more potent than isoflavones in inhibiting aromatase (Kao et al., 1998).

Our results from aromatase assays in the cell line expressing high levels of aromatase also showed a dose dependent aromatase inhibition that is in agreement with what was observed in human placental microsomes. Higher concentrations were needed to obtain 50% of aromatase inhibition (21 μM) in the cell system in comparison to the

microsomal value. This might be explained by the simplicity of the placental microsomes system, where the enzyme is directly accessible to the inhibitors. In addition, as it has been described that isoflavonoids bind estrogenic receptors, these data may be also explained by the existence of other mechanisms, such as influence of estrogen receptors, growth factor receptors and others.

As we showed that the soya extract has anti-aromatase activity and as it has been described that aromatase inhibitors have antiproliferative activity, it was also investigated its effects on cell morphology and on proliferation of MCF-7aro cells. Morphological studies demonstrated drastic morphological alterations depending on the concentrations used. The lowest concentration showed a slight reduction in cell density; the cultures presented well preserved cell morphology, a few non-adherent round cells and membrane blebbings, a characteristic of apoptotic cell death. The highest concentration of soya extract induced a massive cell death, with almost all the cells detached and abundant cell debris, showing that this concentration was toxic and probably induced necrosis.

Preliminary results from the cell growth inhibition caused by genistein and daidzein showed that the proliferation of the MCF-7aro cells, induced by testosterone at concentrations within physiological range, was inhibited in a dose-dependent manner. In our study we found remarkable differences in cell proliferation between concentrations, while 0,74 μM of total concentration of isoflavones showed only a reduction of 20% of cell proliferation, the concentration of 1,71 μM induced a dramatic reduction in DNA synthesis. These results are in agreement with the observations in contrast phase microscopy and Giemsa staining. Nevertheless, these findings are not in accordance with other studies that demonstrated that isolated isoflavones present a biphasic effect on cell proliferation. In low concentrations (less than 10 μM) induce proliferation, whereas in higher (more than 10 μM) concentrations cause an antiproliferative effect (Brooks and Thompson, 2005). There is an evidence from *in vitro* and some *in vivo* studies that the anticancer activity of daidzein and genistein in breast cancer is mediated through cell cycle arrest and apoptosis (Jin et al., 2010). Daidzein was found to arrest cell cycle at the G_1 and G_2/M phases in human breast cancer cells MCF-7 (Choi and Kim, 2008) and induced apoptosis via the mitochondrial pathway (Jin et al., 2010). Moreover, it has been demonstrated that

genistein also induces a G2/M cell cycle arrest in breast cancer and a dose-dependent decrease in the expression of cyclin B, which plays important roles in the positive regulation of CDK activity (Banerjee et al., 2008). In our study the highest concentration, of approximately 3,5 μM, showed 98% of reduction in cell proliferation suggesting that daidzein and genistein may have a synergestic effect, leading to a more potent cell cycle arrest and/or cell death. Further studies have to be carried out within the range of concentrations used in order to understand the process of cell death.

In summary, our preliminary data strongly suggest that the biotransformed soya extract acted as a potent aromatase inhibitor and has an antiproliferative activity in MCF-7aro cells. This work can open new research avenues for the elucidation of the mechanisms associated with the reduction of breast cancer cell growth, and may contribute to the potential use of these type of natural compounds for the treatment of breast cancer. However, further studies with increased number of experiments and proper statistical analysis are required for definitive conclusions.

6. ABBREVIATIONS

AF1	transcriptional activation function 1
AF2	transcriptional activation function 2
AP-1	trancription factor controling cell differentiation, proliferation and apoptosis
BRCA1	human tumor suppressor gene type 1
BRCA2	human tumor suppressor gene type 2
cAMP	cyclic andenosine monophosphate
cDNA	copy deoxyribonucleic acid
CFBS	charcoal-stripped heat-inactivated fetal bovine serum
DBD	DNA-binding domain
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine tetraacetic acid
EGFR	epidermal growth factor receptor
ERE	estrogen responsive element
ERs	estrogen receptors
ER-α	estrogen receptor α
ER-β	estrogen receptor β
FBS	fetal bovine serum
GFRs	growth factor receptors
HDL	high density lipoproteins
HER2	human epidermal growth factor receptor 2
IGFR1	insulin-like growth factor receptor 1
LDB	ligand binding domain
LDL	low density lipoproteins

MAPK	mitogen-activated protein kinase
MEM	minimum essential medium
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NF-κB	nuclear factor kappa B
PI3K	phosphatidylinositol 3-kinase
PTK	tyrosine protein kinase
TCA	trichloracetic acid

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